In Vitro Synthesis of Lysozyme by Human and Mouse Tissues and Leucocytes

D. B. L. McClelland* and R. van Furth

Department of Microbial Diseases, University Hospital, Leiden, The Netherlands

Summary. Previous studies have shown that lysozyme can be detected in many body fluids, in extracts of tissues, and also in granulocytes, monocytes and macrophages. However, the sites of synthesis of lysozyme have not been defined. In the present report, the synthesis of lysozyme by tissues, and by defined cell populations cultured *in vitro* has been studied by detecting the incorporation of ^{14}C-labelled amino acids into lysozyme. This method detects only lysozyme newly synthesized during incubation of the specimen and therefore shows unequivocally which tissues and cell types are capable of lysozyme synthesis. The validity of the method has been shown by parallel studies using an independent method to detect lysozyme production *in vitro*.

In studies in humans and mice, lysozyme synthesis has been demonstrated in the mucosa of the respiratory and gastrointestinal tracts, and in lymphoid organs. In studies of defined cell populations, monocytes and macrophages (mononuclear phagocytes) have been shown to synthesize lysozyme. Granulocytes from peripheral blood contain lysozyme but do not synthesize it, and lymphocytes neither contain nor synthesize lysozyme.

The present findings provide further evidence that lysozyme has an important role in the defence of the host against micro-organisms, and the findings suggest that lysozyme may reach its target by several routes. At an intracellular level it is delivered from lysosomes into the phagocytic granules of granulocytes and macrophages. Local synthesis of the mucous membranes contributes lysozyme to secretions. Synthesis and secretion by mononuclear phagocytes which reach a tissue in response to an inflammatory stimulus contribute lysozyme to the exudate, and the release of lysozyme from breakdown of granulocytes has the same effect.

INTRODUCTION

Lysozyme was discovered by Fleming as a result of its remarkable bacteriolytic effect (Fleming, 1922). It is now known that enzymes with the same substrate are found in many species, although there are major differences in the composition of the lysozymes of different species (Imoto, Johnson, North, Phillips and Rupley, 1972). The antibacterial action of lysozyme has been extensively reviewed (Glynn, 1968; Imoto *et al.*, 1972). When lysozyme acts alone, its most striking activity is against nonpathogenic organisms.

* Present address: University Department of Therapeutics, Royal Infirmary, Edinburgh.

Correspondence: Dr R. van Furth, Department of Microbial Diseases, University Hospital, Leiden, The Netherlands.

However, its substrate is the mucopeptide (Mandelstam and Rogers, 1959), which is a constituent of the cell walls of many bacteria, including mycobacterial strains (de Wijs and Jolles, 1964). It has been shown that bacteria can be made sensitive to lysozyme in various ways, such as treatment with EDTA (Repaske, 1956) or culture in sublethal concentrations of penicillin (Warren and Gray, 1963) and it has recently been shown (Lahav, Ne'eman, Adler and Ginsburg, 1974) that lysozyme can augment the degradation of staphylococcal cell walls caused by leucocyte enzymes. Lysozyme also acts with serum antibody and complement to produce lysis or killing of bacteria (Wardlaw, 1962; Glynn and Milne, 1965; Muschel, Carey and Baron, 1959), and can co-operate with secretory IgA antibodies, in the presence of complement or other serum factors to lyse *E. coli* (Adinolphi, Glynn, Lindsay and Milne, 1966; Burdon, 1973; Hill and Porter, 1974).

Lysozyme is also involved in the destruction of bacteria by phagocytic cells. During phagocytosis of bacteria by polymorphs or macrophages, lysozyme is liberated from the cell granules (Cohn and Hirsch, 1960; Cohn and Wiener, 1963) and it has been shown that while lysozyme-sensitive bacteria are rapidly broken down following phagocytosis, resistant strains of the same organism are much less rapidly lysed (Brumfitt and Glynn, 1961). The importance of lysozyme in polymorphs is illustrated by a recent report of a defect in bacterial killing by leucocytes in which a selective lack of lysozyme and trans-ferrin was demonstrated (Spitznagel, Cooper, McCall, Dechatelet and Welsh, 1972).

Lysozyme is widely distributed throughout the body. Many body fluids contain it, in particular tears and breast milk, which may contain very high concentrations (Fleming and Allison, 1922; Jolles and Jolles, 1967). Extracts of many tissues were shown by early workers to contain lysozyme (Florey, 1930; Fleming, 1932) and more recently studies with immunofluorescence (Glynn and Parkman, 1964; Assamer, Schmalzl and Braun-steiner, 1969; Braunsteiner and Schmalzl, 1971) and histochemical methods (Briggs, Perillie and Finch, 1966; Syren and Raeste, 1971) have demonstrated the presence of lysozyme in monocytes, macrophages and granulocytes. Klockars and Osserman (1974) have used an immunoperoxidase method to show lysozyme in the proximal renal tubules, Paneth cells, and macrophages of the rat. However, none of these methods provides evidence about the sites of synthesis of lysozyme.

In the present study, a technique has been used which allows the detection and identi-fication of proteins synthesized in *in vitro* cultures of cell suspensions or tissue fragments. The ability of tissues and of defined cell populations to produce lysozyme has been studied with the aim of gaining information about the localization of lysozyme synthesis.

MATERIALS AND METHODS

Incorporation of ^{14}C-labelled amino acids into lysozyme in vitro

Newly synthesized lysozyme in cultures of cell preparations or tissues was detected by the incorporation of ^{14}C-labelled amino acids into lysozyme, using a modification of the method of Hochwald, Thorbecke and Asofsky (1961). Fragments of tissue minced with scalpel blades, bone marrow flakes, cell suspensions, or glass-adherent cells were in-cubated for various periods in modified Eagle's medium containing 1 μCi/ml of ^{14}C-L-lysine (specific activity 310 mCi/mmole) and 1 μCi/ml of ^{14}C-L-isoleucine (312 mCi/mmole; Schwarz Bio-research, Orangeburg, New York). The medium con-tained penicillin (100 u/ml) and when contaminated tissues such as intestinal mucosa were cultured, gentamycin (25 μg/ml) and nystatin (75 u/ml) were also added. After

incubation, the cultures were frozen at $-20°$ and after thawing were centrifuged for 20 minutes at 20,000 *g* and dialysed against 0·015 M phosphate buffer, pH 7·6, to remove the unincorporated amino acids, using Visking 8/32 inch dialysis tubing (Visking division, Union Carbide International Company, New York).

The dialysed culture fluids were concentrated approximately 10-fold by freeze drying and redissolving in distilled water. The concentrated culture fluids were tested for the presence of labelled lysozyme by immunoelectrophoresis in 1·5 per cent agarose (L'industrie Biologique Française, Gennevilliers, France) using 0·1 M veronal buffer (pH 8·6), followed by autoradiography of the dried slides.
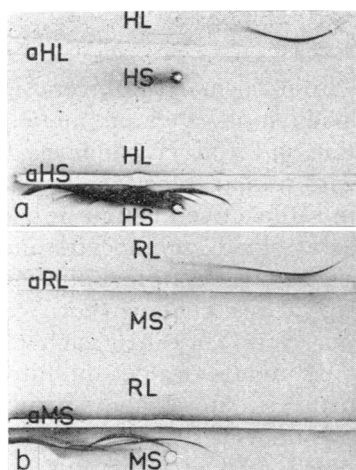

FIG. 1. Immunoelectrophoresis in agarose, developed with (a) anti-human lysozyme and (b) anti-rat lysozyme antiserum. HL = human lysozyme; HS = human serum; RL = rat lysozyme; MS = mouse serum; aHl, aHS = anti-human lysozyme, anti-human serum; aRL = anti-rat lysozyme; aMS = anti-mouse serum. The anti-lysozyme sera show no reaction with whole serum.

Since many of the concentrated culture fluids did not contain enough lysozyme to give a good precipitin line, a carrier was routinely used. For mouse cultures, the carrier was a purified rat lysozyme and the antiserum was a rabbit anti-rat lysozyme. This is an appropriate system to employ for the co-precipitation of mouse lysozyme because there is a high degree of immunological cross-reactivity between rat and mouse lysozymes as shown by Glynn and Parkman (1964). The carrier used for human cultures was lysozyme purified from monocytic leukaemia urine, and the rabbit anti-human lysozyme used was obtained commercially (Dakopatts, Brostex A/S, Copenhagen, Denmark). Fig. 1 shows the immunoelectrophoretic patterns obtained with these antisera.

The cultures were also tested for synthesis of serum proteins by immunoelectrophoresis and autoradiography (van Furth, Schuit and Hijmans, 1966a). Normal mouse and human sera were used as carriers. Anti-human serum was obtained from the Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands, and anti-mouse serum, prepared in rabbits, was kindly provided by Dr Keith James, Department of Surgery, Edinburgh University.

The intensity of the autoradiographs was scored on an arbitrary scale from (+) (just visible) to + + + + (a strong black line) and the mean scores were calculated by assigning values from 1 = (+) to 5 = + + + +.

Tissues

Human tissues were obtained during routine surgery and diagnostic biopsy and processed immediately. With the exception of parotid gland tissue, which was obtained during the excision of parotid tumours, only histologically normal tissues were included in this study. The fragments of tissue which were cultured weighed 50–200 mg with the exception of conjunctival biopsies which weighed 25–30 mg.

Mouse tissues were obtained from male specific pathogen-free Swiss mice weighing 25–30 g (Central Institute for Breeding of Laboratory Animals TNO, Bilthoven, The Netherlands). The specimens were taken immediately after the animals had been killed with chloroform and processed immediately.

Peripheral blood

Human blood was obtained from normal volunteers. Mouse blood was collected by cardiac puncture. Peripheral blood lymphocytes and monocytes were isolated from human and mouse blood by centrifugation on Ficoll–Hypaque, as described by Böyum (1968).

In some of the experiments with human blood, cultures containing both lymphocytes and monocytes were cultured in radioactive medium in roller tubes for 48 hours.

Further separation of peripheral blood lymphocytes and monocytes was obtained by culture on glass coverslips in Leighton tubes in medium 199 (Microbiological Associates, Bethesda, Maryland) containing 20 per cent newborn calf serum (Grand Island Biological Company, Grand Island, New York). In each experiment the cell suspension was adjusted to contain $0 \cdot 5$–2×10^6 monocytes per millilitre. One-millilitre aliquots were incubated in separate Leighton tubes. After 2 hours incubation to allow cells to adhere to the glass surface, the supernatant cell suspensions (morphologically 95–99 per cent lymphocytes) were removed, pooled, washed and cultured in 1 ml of radioactive medium in a roller tube. The glass-adherent cells were washed twice with medium 199 and incubated for a further 24 hours before being washed and having labelled medium added. At the end of the culture period the tubes were frozen at $-20°$ and pooled before further processing. The total numbers of cells cultured in each experiment are given in Tables 1 and 2.

Normal human granulocyte suspensions were obtained by taking the pellet of granulocytes and erythrocytes obtained after centrifugation of whole blood on Ficoll–Hypaque. After being washed, the cells were resuspended to the original blood volume with physiological saline and mixed with 5 per cent dextran (molecular weight 200,000) (Pharmacia, Upsala, Sweden) to sediment the red cells (van Furth *et al.*, 1966a). The cells in the supernatant (95–98 per cent granulocytes) were washed and cultured in radioactive medium in a roller tube.

Peripheral blood leucocyte suspensions from patients with monocytic, lymphocytic or myeloblastic leukaemia were isolated by sedimentation with high molecular weight dextran.

Human thoracic duct lymphocytes were obtained from patients undergoing thoracic duct drainage. The latter cultures were prepared in the course of a previous study on immunoglobulin synthesis (van Furth, Schmit and Hijmans, 1966b).

Bone marrow

Specimens of normal human sternal and rib bone marrow were obtained from patients undergoing thoracic surgery. Cultures of whole marrow were made as described above.

Mouse bone marrow was obtained as described by van Furth and Cohn (1968). Cell suspension containing $1-2 \times 10^7$ nucleated cells per millilitre were incubated for 2 hours in medium 199 with 20 per cent newborn calf serum in Leighton tubes with flying cover-slips. After 2 hours of culture on glass, the supernatant cells were removed, pooled, washed and cultured in radioactive medium in a roller tube and the adherent cells were washed. In one experiment labelled medium was added immediately, otherwise the glass-adherent cells were cultured for a further 24 or 48 hours before being washed again and given the labelled medium.

Skin macrophages

Human skin macrophages were obtained from normal subjects by the 'skin window' technique (Rebuck and Crowley, 1955). A small abrasion was made on the flexor aspect of the forearm and covered with a 10×35 mm coverslip. The coverslips were left *in situ* for 4 hours and then replaced with a new coverslip for a further 18 hours. After removal, each coverslip was immediately placed in a Leighton tube containing medium 199 with 20 per cent newborn calf serum. After washing twice to remove non-adherent cells, the coverslips were incubated for a further 48 hours in radioactive medium.

Mouse skin macrophages were obtained by subcutaneous implantation of glass cover-slips for 24 hours (Volkman and Gowans, 1965). After this period of implantation each coverslip carries approximately 10^6 adherent macrophages. The coverslips were placed in Leighton tubes and incubated in radioactive medium.

Peritoneal macrophages

Mouse peritoneal macrophages were harvested and cultured according to van Furth and Cohn (1968). Cell suspensions containing $1-2 \times 10^6$ cells per millilitre were incubated in Leighton tubes. After 2 hours incubation the supernatant cells were removed, pooled, washed and incubated in a roller tube with radioactive medium. The glass-adherent cells were washed twice and incubated for a further 24 or 48 hours in radioactive medium.

Alveolar macrophages

Mouse alveolar cells were obtained by washing out the lungs with physiological saline containing 0.2 mg/ml of EDTA. The cell suspensions, containing 0.5×10^6 cells per milli-litre were incubated in Leighton tubes in medium 199 with 20 per cent newborn calf serum for 2 hours, then washed and incubated for a further 48 or 96 hours in radioactive medium.

RESULTS

CONTROL EXPERIMENTS

Viability of cultures

Viability of all cultures was checked by the incorporation of label into serum proteins, as detected by autoradiography after immunoelectrophoresis with anti-whole mouse or human serum and normal serum carrier. Granulocyte cultures showed only weak labelling of an unidentified line with β-mobility. However, by testing for trypan blue exclusion, these cells were shown to be 90–95 per cent viable at the start of the period of incubation and after 24 hours in culture. Ninety-three per cent of the cultured granulocytes were

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capable of phagocytosing opsonized *Staphylococcus albus*, using the method described by van Furth and van Zwet, 1973.

Non-specific adsorption of isotope

To test the possibility that the ^{14}C-labelled amino acids in the culture medium could bind non-specifically to lysozyme of the cultured tissue, or to the carrier, 100 μg of rat lysozyme and 100 μg of human lysozyme were each incubated separately for 48 hours with 1 ml of the labelled culture medium. The medium from each experiment was then dialysed, concentrated and tested by immunoelectrophoresis and autoradiography. No labelling of the lysozyme line was detected. Further evidence against non-specific binding of label to lysozyme is provided by the fact that granulocyte cultures, which contained large amounts of unlabelled lysozyme (Fig. 2) never showed any labelling of the lysozyme line.
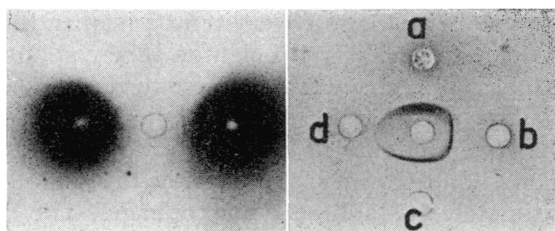

FIG. 2. Lysozyme in human granulocyte and monocyte cultures. Right-hand side: stained plate. Left-hand side: autoradiograph. (a) Granulocyte culture; (b) monocyte culture plus lysozyme carrier; (c) lysozyme carrier alone; (d) monocyte culture alone. Despite the large amount of lysozyme in the granulocyte culture (a), there is no labelling of the precipitin line. The monocyte culture (b, d) contains insufficient total lysozyme to give a clear precipitin line, but when a carrier is used (b) the line is clearly labelled. Labelling is also faintly visible at (d) where the precipitin lines from wells (a) and (c) merge, due to co-precipitation of the labelled lysozyme.

Effect of inhibitor of protein synthesis

The effect of adding an inhibitor of protein synthesis to the culture system was also investigated. A pooled suspension of mouse peritoneal cells was distributed equally among four Leighton tubes, and incubated with ^{14}C-labelled medium containing increasing concentrations of cycloheximide from 1 to 10 μg/ml. Fig. 3 shows the progressive reduction in incorporation of label with increasing cycloheximide concentrations.

Demonstration of lysozyme synthesis by an independent assay

Lysozyme has been detected by most workers by measuring bacteriolytic activity either in a suspension of organisms, or in a gel-diffusion assay (the 'lysoplate', described by Osserman and Lawlor, 1966). Since the method used in the present study depends on the precipitation of radioactively labelled lysozyme by antibody, it was considered important to demonstrate that comparable results could be obtained with the two types of assay. For this experiment mouse peritoneal macrophages were used since these were conveniently obtained and showed active lysozyme synthesis by the ^{14}C incorporation method.

Mouse peritoneal cells (1.5×10^7) were cultured in a lysozyme-free medium (medium 199 with 1 per cent newborn calf serum), which was changed at intervals. The medium removed at each change was divided into two parts, one of which was dialysed for 24 hours against phosphate buffer, 0·015 M, pH 7·6. Both aliquots were then concentrated
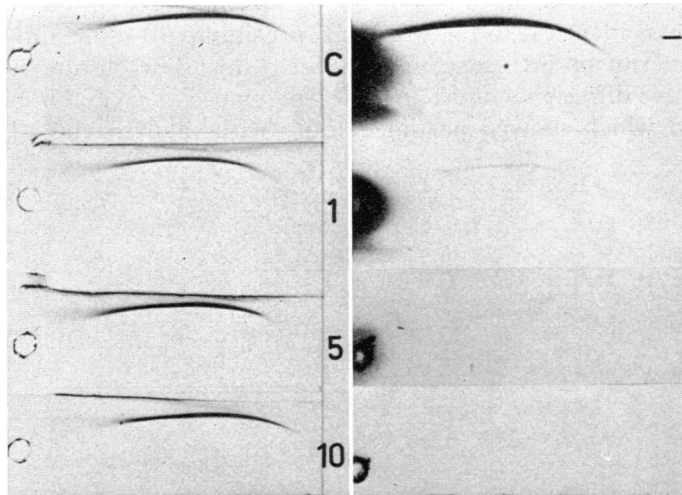
FIG. 3. Inhibition by cycloheximide of lysozyme synthesis by mouse macrophages. Left-hand side: stained slides. Right-hand side: autoradiographs. c = Control preparation; 1 = cycloheximide (1 μg/ml); 5 = cycloheximide (5 μg/ml); 10 = cycloheximide (10 μg/ml).
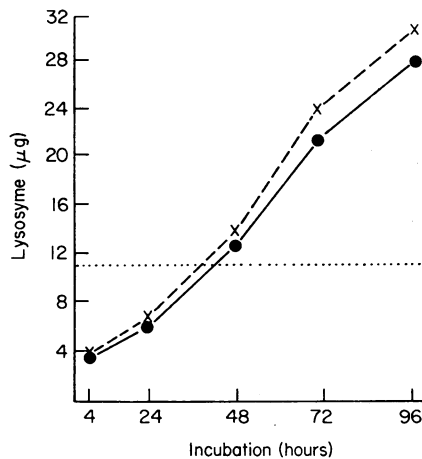

FIG. 4. *In vitro* synthesis of lysozyme by mouse peritoneal macrophages. The horizontal dotted line indicates the lysozyme content of the initial cell suspension, the curves show the cumulative output of lysozyme into the medium tested after dialysis and concentration (●——●) and after concentration only (× — — ×).

by freeze drying. The lysozyme activity in the concentrated medium was then measured by the lysoplate assay, using rat lysozyme standards. As Fig. 4 illustrates, there was a continuous release of lysozyme into the medium, which after 96 hours of incubation represented almost three times the total lysozyme content of the initial cell suspension (measured on an extract made of an aliquot of the initial suspension disrupted by four cycles of freezing and thawing). This observation is in agreement with the findings of Gordon, Todd and Cohn (1974) who also demonstrated that cultured mouse peritoneal macrophages continuously release lysozyme into the medium.

The experiment described above does not prove that the substance causing lysis in the lysoplate assay is identical to the substance precipitated by the anti-lysozyme serum, and a further experiment was done to investigate this. The culture supernatants were first tested by double diffusion against anti-rat lysozyme (Fig. 5) and a sharp precipitation line was obtained which showed partial identity with rat lysozyme. The concentrated
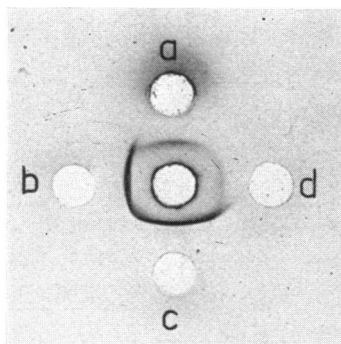

FIG. 5. Demonstration of lysozyme in supernatant of mouse peritoneal macrophage culture. Centre well = anti-rat lysozyme. a and d = Concentrated supernatants; b and c = purified rat lysozyme. The concentrated supernatants give a precipitin line which shows partial identity with rat lysozyme.
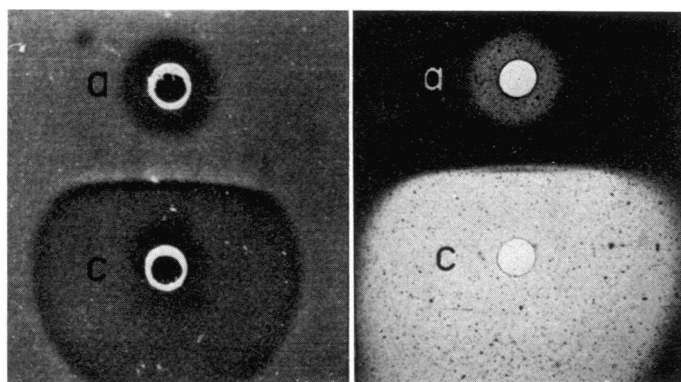

FIG. 6. Inhibition of the bacteriolytic activity in the supernatant of a culture of mouse peritoneal macrophages by anti-lysozyme antiserum. The gel contains killed *Micrococcus lysodeikticus*. a = Anti-rat lysozyme; c = culture supernatant. On the left is the unstained plate, photographed after 18 hours diffusion, showing inhibition of lytic activity in the zone where antiserum is present. On the right is the same plate which has been stained after a further 24 hours diffusion. The precipitin line between lysozyme and anti-lysozyme corresponds in position with the zone of inhibition of lysis, indicating that all the lytic activity in the supernatant was due to lysozyme.

supernatant of a peritoneal cell culture was then placed in one well of a 'lysoplate', with antiserum to rat lysozyme (absorbed with bentonite to remove its intrinsic lysozyme activity) in the adjacent well (Fig. 6). The plate was photographed after 18 hours at room temperature, at which time there was a clear inhibition of lysis in the region of the antiserum. The plate was then allowed to diffuse for a further 24 hours, washed in physiological saline, and stained with amido black. The stained preparation clearly shows that the inhibition of lysozyme activity corresponds closely with the precipitin

line formed between the lysozyme and antiserum, demonstrating that the lytic activity is due to lysozyme only.

Dialysis of lysozyme

The observations of Craig, King and Stracher (1957) indicate that lysozyme (molecular weight 14,700) would not be expected to pass through the dialysis membrane under the experimental conditions used. However, to test this possibility preliminary experiments were done in which human and rat lysozyme, dissolved in culture medium, were dialysed against small volumes of phosphate buffer for 24 hours. No lysozyme was detectable in the dialysis fluid after 20-fold concentration. The experiment illustrated in Fig. 4 also illustrates the small effect of dialysis on the lysozyme activity in the culture medium, showing that under the conditions employed, there is little loss from the dialysis sac.

LYSOZYME SYNTHESIS IN HUMAN TISSUES

Lymphoid organs (Table 1)

Six of the seven spleen cultures showed weak lysozyme synthesis, as did two of the eight lymph node cultures and two of the six cultures of tonsil tissue. Thymic biopsies from eight individuals were cultured, and in no case was lysozyme synthesis detectable. All these tissues were shown to have synthesized immunoglobulins in culture.

TABLE 1

In vitro SYNTHESIS OF LYSOZYME BY HUMAN AND MOUSE LYMPHOID TISSUES

	Number of cultures tested	Number of positive cultures	Mean intensity of labelling of autoradiographs of positive cultures
Human			
Lymph node	8	2	(+)
Spleen	7	6	(+)
Thymus	6	0	—
Tonsil/adenoid	6	2	(+)
Mouse			
Spleen	7	6	+

Respiratory tract (Table 2)

Lysozyme was synthesized by nasal mucosa, trachea (Fig. 7), bronchus and lung cultures. Synthesis was very strong in the tracheal cultures.

Gastrointestinal tract (Table 3)

Synthesis was found in all parotid gland and gastric mucosal cultures and in three of four cultures of duodenal mucosa, although in these cultures labelling was much weaker.

In jejunal and rectal biopsies only occasional very weak synthesis was found. Only two liver biopsies have been cultured; neither synthesized lysozyme. All the gastrointestinal biopsies showed active synthesis of immunoglobulin (mainly IgA) and the liver biopsies synthesized other non-immunoglobulin proteins.

Skin and other mucous membranes

No synthesis of lysozyme was found in biopsies of normal skin, conjunctival mucosa or vaginal mucosa.

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TABLE 2

In vitro SYNTHESIS OF LYSOZYME BY HUMAN AND MOUSE RESPIRATORY SYSTEM TISSUES

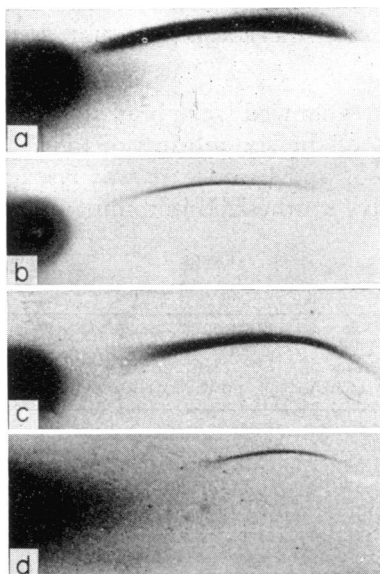|	Number of cultures tested	Number of positive cultures	Mean intensity of labelling of autoradiographs of positive cultures
Human			
Nasal mucosa	4	3	+ +
Trachea	4	4	+ + +
Bronchus	2	2	+ +
Lung	4	3	+
Mouse			
Trachea	3	3	+ +
Lung	5	4	+ +

FIG. 7. Examples of autoradiographs of immunoelectrophoretic patterns, showing labelled lysozyme arc. The cultures illustrated are: (a) mouse peripheral blood monocytes; (b) mouse bone marrow macrophages; (c) human tracheal mucosa; (d) normal human peripheral blood monocytes.

LYSOZYME SYNTHESIS IN MOUSE TISSUES (Tables 1–3)

Six out of seven cultures of mouse spleen synthesized lysozyme. All cultures of trachea, and four out of five cultures of lung were also positive.

The findings in mouse gastrointestinal tissues were quite different from those in man. No lysozyme synthesis was found in stomach nor in the mucosa of small or large bowel, the only exception being very weak synthesis in occasional cultures of Peyer's patch tissue. Mouse liver, like human, did not synthesize lysozyme. In four cultures of normal mouse skin, no lysozyme synthesis was detected.

LYSOZYME SYNTHESIS BY HUMAN LEUCOCYTES (Table 4)

Cultures of a mixed population of human peripheral blood lymphocytes and mono-

cytes (about 40 per cent monocytes) synthesized labelled lysozyme. This synthesis was entirely attributable to the glass-adherent cells, because after settling on glass, the monocytes showed active synthesis (Fig. 7), whereas the isolated lymphocytes did not synthesize lysozyme, despite the fact that these latter cultures synthesized immunoglobulins.

TABLE 3

In vitro SYNTHESIS OF LYSOZYME BY HUMAN AND MOUSE GASTROINTESTINAL TISSUES

	Number of cultures tested	Number of positive cultures	Mean intensity of labelling of autoradiographs of positive cultures
Human			
Oral mucosa	4	0	—
Parotid gland	3	3	+ +
Stomach (antrum)	9	9	+ +
Duodenum	4	3	+
Jejunum	7	2	(+)
Rectum	14	2	(+)
Liver	2	0	—
Mouse			
Stomach (body)	2	0	—
(fundus)	2	0	—
Jejunum	7	0	—
Peyer's patch	6	5	(+)
Large intestine	6	0	—
Liver	6	0	—

TABLE 4

LYSOZYME SYNTHESIS BY HUMAN LEUCOCYTES

Type of cells	Number of cells per culture	Number of cultures tested	Time period of incubation in labelled medium (hours)	Number of cultures which synthesized lysozyme	Mean intensity of labelling of autoradiograph*
Normal peripheral blood					
lymphocytes plus monocytes	$2–7 \times 10^7$	6	0–48	6	+ +
Separated peripheral blood					
Monocytes	$1·1,1·8 \times 10^7$	2†	2–26	2	+ +
Lymphocytes	$2–4 \times 10^7$	3	2–26	0	—
Granulocytes	$4–8 \times 10^7$	4	2–26	0	—
Bone marrow		6	0–48	6	+
Skin macrophages	$0·5–1 \times 10^6$	2	0–48	2	(+)
Thoracic duct lymphocytes	1×10^8	3	0–48	0	—
Peripheral blood leucocytes from:					
Monocytic leukaemia	$0·3–1 \times 10^8$	6	0–48	6	+ +
Chronic lymphatic leukaemia	$1–6 \times 10^8$	8	0–48	0	—
Acute myeloblastic leukaemia	$2–3 \times 10^8$	6	0–48	6	+

* Calculated as described in the text under the Materials and Methods section.
† Cultures of peripheral blood monocytes in Leighton tubes were pooled as described under the Materials and Methods section in two separate experiments. The numbers given are the total monocytes represented by the pooled cultures from each experiment.

Human thoracic duct lymphocytes obtained during thoracic duct drainage also synthesized immunoglobulin but not lysozyme.

Cultures of whole human bone marrow showed active synthesis of lysozyme.

Skin macrophages were obtained by the skin window method from two subjects and lysozyme synthesis was found in cultures of these preparations.

Cultures of peripheral blood leucocytes (containing about 70 per cent monocytes) obtained from monocytic leukaemic patients invariably showed lysozyme synthesis, whereas cultures of leucocytes from chronic lymphatic leukaemia (about 90 per cent lymphocytes) never did. Cultures from patients with myeloblastic leukaemia contained 60–90 per cent blast cells and 0–1 per cent of monocytes. Lysozyme synthesis was seen in these cultures, and in some cases may be attributable to the small percentage of monocytes present $(2-3 \times 10^6$ monocytes per culture).

Synthesis of lysozyme was never found in cultures of normal human peripheral blood granulocytes. However, these culture fluids contained large amounts of unlabelled lysozyme demonstrable by double diffusion as shown in Fig. 2, and by immunoelectrophoresis.

TABLE 5

LYSOZYME SYNTHESIS BY MOUSE LEUCOCYTES

Type of cells	Number of animals used to provide cells for each experiment	Total number of cells cultured in each experiment*	Time period of culture in labelled medium (hours)	Mean intensity of labelling†
Peripheral blood monocytes	4	7×10^5	2–26	+
	15	3×10^6	26–74	+ + + +
	15	3×10^6	74–120	+ + + +
Peripheral blood non-adherent cells	15	1×10^7	2–50	(+)
Bone marrow mononuclear phagocytes	3	1×10^6	2–26	+ + +
	3	1×10^6	26–50	+ + + +
	3	1×10^6	50–74	+ + + +
Bone marrow non-glass-adherent cells	3	6×10^7	2–26	−‡
	3	6×10^7	2–26	(+)
	9	2×10^8	2–50	+
Peritoneal macrophages and lymphocytes	4	1×10^7	0–48	+ +‡
Peritoneal macrophages	4	1×10^7	2–26	+‡
	4	1×10^7	2–50	+ + + +
Peritoneal lymphocytes	4	1×10^6	2–26	−‡
	4	1×10^6	2–50	−
Alveolar macrophages	20	1×10^6	2–50	+ + + +
	20	1×10^6	50–98	+ + + +
Skin macrophages	10	1×10^7	2–50	+ + + +
	10	1×10^7	50–98	+ + + +

* The number of cells cultured in each experiment is the total representing the pooled cultures from that experiment.
† Calculated as described in the text under the Materials and Methods section.
‡ Mean of three experiments.

LYSOZYME SYNTHESIS BY MOUSE LEUCOCYTES (Table 5)

Mononuclear phagocytes (monocytes and macrophages) obtained from peripheral blood (Fig. 7), bone marrow, peritoneal cavity, lung and skin all showed active synthesis of lysozyme. Although synthesis was detectable in the first 24 hours, the amount of labelled lysozyme increased to a higher, constant level in cultures which were maintained for longer periods before addition of the ^{14}C-labelled medium.

Lysozyme synthesis was weakly detectable in some cultures of the non-glass-adherent cells of bone marrow, although these cultures contained much larger numbers of cells than the corresponding glass-adherent cell cultures (Fig. 7). The mixed population of

cells in these cultures has not been characterized. A culture of the non-glass-adherent population of peripheral blood leucocytes separated on Ficoll–Hypaque also showed weak synthesis of lysozyme. Although this suspension consisted mainly of lymphocytes (approximately 98 per cent) 1–2 per cent of monocytes were present as a result of incomplete glass-adherence, and are presumed to account for the observed lysozyme synthesis.

DISCUSSION

This study shows that synthesis of lysozyme *in vitro* can be demonstrated by detecting the incorporation of ^{14}C-labelled amino acids. Using this method it has been shown in man and mice that lysozyme is synthesized by tissues of the gastrointestinal and respiratory tracts, by lymphoid organs, and by cells of the mononuclear phagocyte system. Neither granulocytes nor lymphocytes from peripheral blood synthesized lysozyme.

These observations may be compared with the results of earlier studies in which lysozyme has been assayed after extraction from cells or tissues, or demonstrated histologically. Several tissues, such as lung and human gastric mucosa, which have been shown to contain large amounts of lysozyme (Florey, 1930) were found to synthesize it actively. Tissues such as rodent stomach and human large intestine, which contain little lysozyme (Florey, 1930) did not synthesize it *in vitro*. Although both mononuclear phagocytes and granulocytes have repeatedly been shown to contain lysozyme (Briggs *et al.*, 1966; Assamer *et al.*, 1969; Syren and Raeste, 1971), only the mononuclear phagocytes have the ability to synthesize the enzyme *in vitro*; mature granulocytes do not synthesize lysozyme. Lymphocytes do not contain lysozyme (Briggs *et al.*, 1966) and do not synthesize it. These findings are supported by the studies on peripheral blood leucocytes of patients with leukaemia. Cultures obtained from patients with acute monocytic leukaemia invariably showed strongly positive lysozyme synthesis, whereas this was never found in patients with lymphatic leukaemia. The findings of lysozyme synthesis by leucocytes from patients with myeloblastic leukaemia suggests that the immature myeloid cells in these patients synthesized lysozyme, but the experiments do not exclude the possibility that the small number of monocytes in the cultures were responsible. Further studies are required in which blast cells are cultured in the absence of monocytes.

Previous studies have demonstrated *in vitro* synthesis of lysozyme by rat bone marrow macrophages (Chakhava and Gorynova, 1965), rabbit alveolar macrophages (Heise and Myrvik, 1967) and leucocytes from patients with monocytic leukaemia (Ohta, Kamiya and Nagase, 1971). All those studied, however, have been based on the assay of lysozyme solely by its ability to lyse sensitive organisms, and little evidence has been presented to demonstrate that the activity detected was solely due to lysozyme. Gordon *et al.* (1974) also using the bacteriolysis assay, have demonstrated synthesis of lysozyme by mouse and human mononuclear phagocytes and shown that cultures of these cells incorporate ^{14}C into a molecule which co-electrophoreses with lysozyme in polyacrylamide gel.

The method used in the present study has the advantage of providing an immunologically specific identification of newly synthesized lysozyme in each culture and avoids difficulties in interpretation arising from the presence of pre-existing lysozyme in the tissues studied. An additional advantage of the technique is the possibility of detecting

the synthesis of other proteins in a single culture of a small specimen, by use of appropriate antisera and carriers.

Significance of lysozyme synthesis by tissues

The finding of lysozyme synthesis by tissues of the gastrointestinal and respiratory tracts indicates that local synthesis in the mucous membranes may account for much of the lysozyme detected in secretions, and that is not, as has been suggested (Lorenz, Korst, Simpson and Musser, 1957) mainly of salivary origin. The present study does not provide any indication as to the cellular localization of lysozyme synthesis in mucous membranes.

In the rat and mouse, lysozyme has been demonstrated in the Paneth cells of the small intestinal crypts by cytochemical methods (Speece, 1964; Ghoos and Vantrappen, 1971) and by immunoperoxidase staining (Klockars and Osserman, 1974). It is not clear how these observations relate to the failure to detect synthesis by mouse small intestine in the present study. In the human parotid gland, Kraus and Mestecky (1971) showed by immunofluorescence that lysozyme was localized in distinct epithelial cells, forming a discontinuous layer at the base of the striated ducts, and these workers concluded that lysozyme was probably synthesized in these cells rather than being concentrated from serum. The findings in the present study confirm that local synthesis occurs in the parotid. Further cytochemical or immunohistochemical studies are required to demonstrate the cells responsible for lysozyme production in the human gastrointestinal tract, and in human and rodent respiratory mucous membranes.

Since it has been shown that gastrointestinal and respiratory mucous membranes are capable of synthesizing immunoglobulin and components of the complement system (Lai A Fat, Suurmond and van Furth, 1973; van Furth and Aiuti, 1969; McClelland, Lai A Fat and van Furth, to be published; Colten, Gordon, Borsos and Rapp, 1968) it is clear that locally synthesized lysozyme could act in conjunction with these other products to generate a highly anti-bacterial microenvironment in the vicinity of the mucous membranes and should perhaps be considered as a part of the secretory immune system.

Significance of leucocyte lysozyme

Mature granulocytes do not synthesize lysozyme. The lysozyme which they contain is involved in the intracellular digestion of bacteria (Cohn and Hirsch, 1960; Glynn and Parkman, 1964), and when the cells die, it is released and contributes to the high levels of lysozyme found in, for example, wound exudates (Senn, Chu, O'Malley and Holland, 1970; McClelland, Raeburn and van Furth, to be published).

Mononuclear phagocytes also contain lysozyme, which is released during the phagocytosis of bacteria (Cohn and Wiener, 1963). However, in addition, these cells actively synthesize lysozyme, so that an accumulation of monocytes and macrophages can lead to the local production of lysozyme in a lesion, even in a tissue such as skin, in which there is normally no synthesis. Since macrophages are also capable of synthesizing components of the complement system (Stecher and Thorbecke, 1967; Lai A Fat and van Furth, 1975), these cells may make an important contribution to the local humoral defences against micro-organisms.

In addition to its role as an antibacterial agent, a recent report indicates that lysozyme has an effect on the growth of tumour cells *in vitro* (Osserman, Klockars, Halper and

Fischel, 1973). This raises the possibility that lysozyme, locally secreted by macrophages, could play a role in the *in vivo* response to tumours.

The findings of lysozyme synthesis by mononuclear phagocytes provides an explanation for the high serum levels of lysozyme which are characteristic of monocytic leukaemia (Osserman and Lawlor, 1966) and which contrast with the lesser elevations found in granulocytic leukaemia (Perillie and Finch, 1973). Elevated serum lysozyme levels are also found in active sarcoidosis (Pascual, Gee and Finch, 1973) and in pulmonary tuberculosis (Khan, Perillie and Finch, 1973). In both these situations large numbers of macrophages accumulate in the granulomatous inflammatory lesion.

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